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THE ACCESSORY FACTORS IN BACTERIAL GROWTH

IV. THE "SATELLITE" OR SYMBIOSIS PHENOMENON OF PFEIFFER'S BACILLUS (*B. INFLUENZAE*)

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If one makes an ordinary blood-agar plate by pouring a tube heavily inoculated with Pfeiffer's bacillus (*B. influenzae*), colonies will appear which are very tiny; often apparently many of the organisms do not grow at all. However, if some other organism, a staphylococcus for example, is inoculated onto the plate and the plate incubated for 24 hours, the Pfeiffer colonies close to the staphylococcus colonies will appear very much larger than those in other parts of the plate and also apparently more numerous. Thus, there appears a prominent central colony (staphylococcus) surrounded by a cluster of relatively large Pfeiffer colonies. This has been termed the "satellite" phenomenon.

In 1897, Grassberger¹ first pointed out that close to the margins of colonies of *Staph. aureus* the Pfeiffer bacillus tended to form giant colonies. He also showed that if the staphylococcus cultures were killed by heat ($\frac{1}{4}$ hour duration) and mixed with blood medium, the growth of the Pfeiffer bacillus was markedly enhanced. He therefore concluded that the bacterial products and not the symbiosis favorably influenced the growth of Pfeiffer's bacillus. In 1898, Meunier² noted the relatively large colonies of Pfeiffer's bacillus about other colonies grown in plate cultures with it, and referred to the phenomenon as "cultural satellitisme." Allen³ observed it on plates when isolating bacteria from certain cases of chronic influenza. He grew Pfeiffer bacilli on medium to which heat killed staphylococcus albus had been added and noted a growth far more profuse than on ordinary blood mediums, attributing it to a toxin highly adjuvant to the growth of Pfeiffer's bacillus. He made identical experiments with *Pneumococcus*, *Staph. aureus*, *B. coli*, *B. acidi-lactici* and *Microc. paratetrigenus*, all of which behaved alike.

The favoring action of organisms on the Pfeiffer bacillus has been observed and noted by many other observers. For years it has been customary to smear the entire slant surface of blood agar with Pfeiffer's bacilli and then make a light streak with staphylococcus

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¹ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 453.

² Semaine méd., 1898, 18, p. 268.

³ Lancet, 1910, 1, p. 1263.

through the center in order to enhance the growth of the former. The favoring action is also evident when plating out material, such as sputum, containing a mixed growth of Pfeiffer's bacillus and other organisms, especially if the former are numerous, in which case the cluster of large Pfeiffer's bacilli colonies about colonies of the other bacteria offer a striking appearance.

Further than to note the phenomenon, little has been done to analyze the factors concerned in its production. For this reason, I have thought the results of certain observations, made to determine its nature, worthy of presentation.

No organism other than Pfeiffer's bacillus is known that behaves in this way when grown with other organisms. At any rate, this statement is true when organisms are tested on blood or hemoglobin plates as described. A somewhat similar appearance is presented at times by certain motile bacteria on plates. A large central colony may become surrounded by a cluster of smaller and younger colonies which have arisen from bacteria moving out from the central colony. This phenomenon can easily be differentiated from the satellitism of Pfeiffer's bacillus because the central colony and those clustered about it are the same organism. Since the Pfeiffer bacillus is unique in this regard the phenomenon has become a most valuable means of differentiating it from other closely related bacteria.

The reaction does not appear to be a mutual one. That is, while the Pfeiffer bacilli are favorably influenced in their growth, the central or foreign colony is not affected either favorably or unfavorably. This may readily be shown by planting a portion of a plate with Pfeiffer bacilli and then inoculating with other bacteria. No difference is noted in the size of the latter colonies in the two halves of the plate. Owing to this fact, the question arises as to whether the term symbiosis should be applied to this phenomenon. Since it is not a mutual process it would seem that the term commensalism would be the proper one to use.

While the favoring action of an organism on the growth of Pfeiffer bacilli on solid blood medium is thus definitely manifested, the same action is not so clear on blood medium when the organisms are intimately mixed and transferred in this condition. Here Pfeiffer's bacilli multiply along with certain feebly growing cocci or bacilli (streptococci, diphtheroids) through several generations, gradually becoming less and less numerous. With *Staph. aureus* it dies out in two or three generations. There would appear to be in mixed culture

another factor inhibiting in character and which quite rapidly overcomes Pfeiffer's bacillus. So, too, in fluid blood cultures Pfeiffer's bacillus may grow through a few generations with a streptococcus or a staphylococcus, soon, however, becoming extinct. Further evidence of an inhibition or antagonistic action of bacteria on Pfeiffer's bacillus will be referred to later. In this connection, too, may be mentioned the fact that on nonhemoglobin medium Pfeiffer's bacillus will live through many generations when growing with another organism. I have grown it mixed with a diptheroid through 7 generations on plain mediums which without this bacillus would not grow at all. Neisser grew it through 20 generations with the *B. xerosis* on plain mediums and others have confirmed these results. The question always enters here as to the possible influence that blood in the medium may have since we now know that heated blood plus bacteria will yield a growth. It would appear, then, that a favorable as well as an unfavorable effect is exerted, by staphylococci at least, and also presumably by other organisms.

The zone of favorable influence about a foreign colony varies considerably in width even for one and the same organism. It may be as narrow as 1 mm. or may be 1 cm. or more in width. It is usually clearly visible for a distance of 2 to 3 mm. There is little difference apparently between the width of the zone of influence about different bacteria. It seems wider when the number of colonies is few.

The zone is symmetrical and most intense usually at or near the margin of the central colony. There are, however, certain exceptions to this. Often about staphylococcus colonies one sees a narrow zone of inhibition which may be a fraction of a millimeter in width. Beyond this the colonies of Pfeiffer bacilli are large and prominent. In this narrow zone the colonies are small or entirely absent. About certain other bacterial colonies, too, zones of inhibition appear which may be very wide. I have a strain of *B. subtilis* which is strongly hemolytic and about which there is a zone of inhibition so wide that it apparently neutralizes the favorable influence that might be exerted by the colony. At any rate, that is the interpretation I have given the observation. The zone of hemolysis is nearly 1 cm. in width; the medium is there alkaline and colonies composed of bacteria other than Pfeiffer bacilli (*Staph. albus*, for instance) are also here inhibited. This is the only organism I have met with thus far that does not reveal a favorable action on the Pfeiffer bacillus; though probably others could be found if systematic tests were made with a great number of bacteria.

When using for central colonies certain bacteria that tend to form large spreading growths, one may observe that the adjacent Pfeiffer colonies may be covered by the spreading margins of the central colony. Hence, one might conclude on superficial examination that such a colony does not act favorably on Pfeiffer's bacillus. However, by more careful inspection one may see the enlarged colonies of Pfeiffer bacilli underneath, or apparently in, the large central colony, showing clearly that a satellite zone was early formed and had later been covered or overgrown by the spreading colony. Strains of *sarcina lutea* and *B. mucosus* will at times behave in this manner.

With the exception of these instances, I have not found an organism that will not enhance the growth of Pfeiffer's bacillus. I have examined the following with positive results: *Staph. albus* and *aureus*, *Strept. hemolyticus* and *viridans*, *Pneumococcus* types 1, 2 and 3, also many strains of group 4, *Meningococcus*, *Micrococcus catarrhalis*, *B. diphtheriae*, *B. pseudodiphtheriae* (many strains of diphtheroids), *Sarcina lutea*, *B. coli*, *B. typhosus*, *B. paratyphosus* (A and B) *B. dysenteriae*, *Sp. metchnikovii*, *gonococcus*, *B. mucosus* (Friedländer), *B. pyocyaneus*, *B. prodigiosus*, *B. sporogenes*, *B. enteritidis*, *B. fecalis-alkaligenes*. Fifteen different organisms were isolated on plates from the air. These were chiefly chromogenic organisms and were not definitely identified. All without exception favorably influenced the Pfeiffer bacilli. I also tested on plates many sputum bacteria, and all colonies that grew revealed the satellite phenomenon with the exception of the strain of *B. subtilis* mentioned. No differences were noted between pathogens and saprophytes.

Strains of yeasts stimulate the growth of Pfeiffer's bacillus very well, provided the strain grows appreciably in 24 to 48 hours. With slow growing organisms in general, the phenomenon is not observed because during the short growth period of Pfeiffer's bacillus the former have not had time to multiply sufficiently to exercise any appreciable influence. This is true of tubercle bacilli, blastomyces, sporotricha and achorion quinckeanum. The tests were all made using at least two strains of Pfeiffer's bacillus, one from a case of influenzal meningitis and the other from a case of bronchopneumonia. In some instances many more strains of Pfeiffer's bacillus were used in the tests.

Pfeiffer bacilli are influenced favorably about to the same degree by both hemolytic and nonhemolytic bacteria. However, there is an advantage in using as a central colony one that causes hemolysis like *Strep. hemolyticus* or a hemolytic colon bacillus because in the clear

zone of hemolysis the Pfeiffer colonies can be readily seen, and their variation in size easily ascertained. Furthermore, the phenomenon is equally well brought out by using blood corpuscles hemolyzed by such agents as water. Satellitism, then, is not dependent on, or due to, hemolysis or to liberation of the hemoglobin from the corpuscles because the phenomenon is observed equally well in blood, unlaked or laked by bacteria or by water.

The reaction is noted on plates about both alkali and acid formers. At least, this is true unless large amounts of these substances are generated. The instance cited of a strain of *B. subtilis* inhibiting the influenza colonies may be an example of a strong alkali former preventing the development of the bacilli as already discussed. On 1% fermentable sugar mediums pneumococci and streptococci still exert their favorable influence.

In connection with the experiments with laked corpuscles some of the blood laked by the addition of a large amount of water was passed twice through a Berkefeld filter. The filtrate, distinctly tinged red and proved to be sterile, was added to the plain medium plates which were inoculated with several strains of Pfeiffer bacilli. Not only did the bacilli grow well on this filtered hemoglobin medium, but the satellite arrangement appeared quite as well as on ordinary blood plates. It is clear from this that the growth factor is not held back or absorbed appreciably by the process of filtration.

The variety of blood used in plating appeared to make no appreciable difference. The phenomenon appears equally well with human, sheep, horse, rabbit, guinea-pig, dog and pigeon bloods. Pigeon blood yields the most profuse growth, and for that reason the satellitism is not so evident.

Washed red cells do as well as unwashed in the medium in revealing the phenomenon. This is an important fact because it excludes serum inhibition as a possible factor in the reaction. Pfeiffer bacilli are to some degree inhibited in their growth by blood serum. Several experiments were made by adding increasing amounts of serum to ordinary chocolate blood medium (heated to 90 C. for 5 minutes), on which an abundant growth appears. As the amount of serum increased, there was noticed some diminution in the amount of growth. In other words, chocolate agar to which unheated serum has been added is not as favorable a culture medium as chocolate agar without the serum. One might postulate that in the zone about the central colony the serum has been altered so that it has lost its inhibiting effect.

While this may or may not be true, it probably has little or nothing to do with this phenomenon since the experiment in which washed corpuscles were used in the medium conclusively demonstrates that the presence of serum inhibition plays no rôle in the reaction.

Experiments were designed to test the influence of other substances on Pfeiffer's bacillus. Masses of dead bacteria killed by heating or drying, or dying naturally in a test tube, were prepared, and a small amount placed on a blood plate seeded with Pfeiffer bacilli. Strains of staphylococci colon bacilli and streptococci, all of which when alive and growing on Pfeiffer plates revealed satellitism, showed no favoring influence when thus tested. This experiment was made many times, and no stimulating effect was ever noted by these dead bacteria. This merely indicates that from the dead bacteria the favorable substance or influence is not disseminated or diffused to any appreciable extent. When dead bacteria or filtrates of cultures are intimately mixed with blood medium, Pfeiffer bacilli will grow on this far more profusely than on medium without the bacteria. From the living and growing bacteria, however, the favorable substance is diffused into the surrounding medium for some distance.

Next sterile animal tissue was tried. Small pieces of guinea-pig and rabbit liver, kidney, myocardium, voluntary muscle, spleen and brain were freshly prepared and placed on a blood plate seeded with Pfeiffer bacilli. About such tissue definite satellitism was seen after 24-hour incubation, all tissues behaving essentially alike in this respect. Curiously enough, a drop of blood placed on a hemoglobin plate does not reveal this reaction, or at least only to a slight degree. For this reason, one can exclude the blood or hemoglobin that may be present in these tissues as the determining factor. When one heats these animal tissues in the autoclave or to boiling for some time, they will no longer show satellitism with Pfeiffer bacilli. So, too, small pieces of fresh carrot and potato obtained under sterile precautions will also reveal the phenomenon on blood plates, but when autoclaved for a short time will no longer do so. One may therefore state that the factor responsible for this satellite phenomenon is destroyed by heat. In all the experiments, of course, proper precautions were taken to avoid any bacterial contamination of the animal or plant tissue. They must be absolutely sterile.

I have tested many chemicals, including iron compounds, both organic and inorganic, oleic acid, etc., without finding one that will consistently do what the living colony or tissue will do. It should be

said that no chemical will act as a substitute for blood or hemoglobin in growing Pfeiffer bacilli.⁴ Ghon and Preyss⁵ showed that on hematin agar Pfeiffer bacilli will grow when associated with another organism but not alone. However, when placed on a blood plate, hematin will not show satellitism. Oleic acid has been added to mediums to advantage in growing Pfeiffer bacilli, especially in the last few years. When small droplets or pieces are placed on a blood plate which has been inoculated with Pfeiffer bacilli, a wide clear zone of hemolysis soon appears around it. Usually the bacilli are not stimulated to grow in this zone, but I have noted at times that near the outer margin of the hemolytic zone large Pfeiffer colonies may form quite comparable to those about a bacterial colony. Whether or not this phenomenon is comparable to that which appears about bacterial growth I am not able to say. Presumably, it changes the hemoglobin into a derivative (hematin), thereby permitting another substance in the blood, serum or in other bacteria to act with it and enhance the growth of the Pfeiffer bacilli. This substance I will discuss further.

The question arises as to the relation of this favoring influence of a foreign organism and the favoring influence of heated hemoglobin on the growth of Pfeiffer bacilli. It has long been known that heating blood medium—that is, chocolate medium—improved it for growing this bacillus. I have studied this phenomenon,⁶ and it appears that in order to obtain the most favorable medium the heating of blood must be done between quite definite limits—55 and 120 C., and for definite periods of time. Blood heated to 55 C. indefinitely will not yield a maximum growth. Heating to 60 C. for 2 to 4 hours is necessary, but if continued for 3 or 4 days, the medium is rendered useless. Boiling the blood medium for a moment will yield a favorable medium, but boiling for 1 to 2 hours will destroy its value for Pfeiffer bacillus cultures. Autoclaving for 30 minutes will also destroy its value. These results may be plotted in the form of a curve.

The fact that superheating or autoclaving will render blood mediums valueless for the culture of Pfeiffer bacillus permits one to analyze the satellite phenomenon further. On an autoclaved blood plate Pfeiffer bacilli will not grow. Mixed with another organism it grows in the zone about the foreign colony very well, thus showing satellitism on the autoclaved medium even better than on unheated blood medium. Moreover, by adding a bacterial filtrate or a carrot or potato filtrate

⁴ Jour. Infect. Dis., 1907, 4, p. 73.

⁵ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 453.

⁶ Jour. Infect. Dis., 1921, p. 169.

(Berkefeld) or blood serum to such filtrated medium, Pfeiffer bacilli will grow profusely, an excellent medium thereby being furnished for the cultivation of this organism.

The appearance and characteristics of the zone on this medium are identical with those described on unheated plates. Owing to the clots of blood formed in the heated medium, in order to observe the satellitism, it is necessary to remove them before plating. This can readily be done by passing the medium through filter paper when hot. Using this filtered blood medium and inoculating with Pfeiffer's bacilli, the phenomenon is satisfactorily observed about a foreign colony or other material used for this purpose.

From this last observation we may conclude that we are dealing with two substances, one of which is heat stabile and is present in the heated blood, the other a substance less heat resistant and found in extracts and filtrates of animal and plant tissues of bacterial cultures. These two substances when operating together in plain medium will allow a profuse growth; either one alone will not permit growth in plain medium. This conclusion has been arrived at and developed in a previous article⁶ through a study of the growth of this organism on autoclaved blood medium in tubes to which various activating substances were added. The results there presented agree entirely with those reported here and enable us to explain satisfactorily the satellite phenomenon. On the autoclaved blood medium plates seeded with Pfeiffer's bacilli growth will appear only around a foreign colony where the heat labile body necessary to complement the heat stabile body of the heated blood is generated. This substance from the foreign colony or from a piece of carrot or animal tissue is a diffusible substance, for it exerts its influence to a distance the width of the satellite zone. On unheated blood plates the process is more complex because, in addition to the hemoglobin and its derivatives, there are other compounds in the serum and even in the corpuscles. It appears, however, that the foreign colony not only furnishes the heat labile substance necessary, but also breaks up the hemoglobin into its derivatives (hematin and hemin) with which the former acts. It is difficult to be certain of this last point because hemoglobin is an unstable substance, and it is difficult to know when it is free from its derivatives. Probably in blood plates there is always some hematin, and it may very well be that pure hemoglobin or oxyhemoglobin will alone (in plain mediums) not support the growth of Pfeiffer bacilli. This would seem to be indicated by the fact that in crystallized hemoglobin medium

or in fresh blood medium the growth of Pfeiffer bacilli is scant and even at times negative. In all probability, then, a foreign bacterium or animal or plant tissue on unheated blood plates breaks down the hemoglobin into its derivative hematin, at least to some extent, and also furnishes the heat labile substance referred to. Thus, in a uniform zone about bacteria, tissue, etc., exist the two substances the cooperation of which is necessary for the profuse growth of Pfeiffer bacilli.

A final point deserving a word of comment is the possible influence of another organism on the growth and possibly on the virulence of Pfeiffer's bacillus in the animal body. The statement has been made that in mixed culture Pfeiffer's bacillus is more dangerous and more pathogenic for animals and possibly for man. Some years ago I made experiments on animals which seemed to indicate that this is true, and it may be true to some degree. However, we now know that various living tissues of the animal will do exactly what an associated organism will do in the way of stimulating the growth of Pfeiffer's bacillus, so that apparently this favoring influence is being exerted by various tissues of the body on this bacillus during an infection, and the additional influence of an associated organism, as for example streptococci or staphylococci in the respiratory tract, would presumably make little difference in the final result.

SUMMARY

The phenomenon of satellitism as observed in connection with Pfeiffer's bacillus is described in detail.

It is observed in association with bacteria, yeasts and fungi of various kinds and their filtrates; also with plant and animal tissues and their extracts and filtrates.

Heating in the autoclave (120 C. for 30 minutes) will destroy the activities of these substances. Heating for longer periods at lower temperatures will do likewise.

On clarified autoclaved blood medium Pfeiffer's bacillus will not grow. On plain medium, to which organisms or tissues or their filtrates (hemoglobin free) are added, it will not grow. When these substances are mixed good growth results.

The explanation of satellitism therefore would seem to be: Diffusible products or extracts of bacteria, fungi, tissues, etc., stimulate the growth of Pfeiffer's bacillus in conjunction with hematin or with hemoglobin. Thus, profuse growth of Pfeiffer's bacilli occurs immediately around colonies of organisms or pieces of plant or animal tissue.